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Original article

The influence of protein malnutrition on biological and immunomodulatory aspects of bone marrow mesenchymal stem cells

Guilherme Galvão dos Santos [a], Shafqat Batool [a, b], Araceli Hastreiter [a], Talita Sartori [a], Amanda Nogueira-Pedro [a], Primavera Borelli [a], Ricardo Ambrosio Fock [a, *]

[a] Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo, 05508-900 São Paulo, SP, Brazil
[b] Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology(NUST), H-12, Islamabad, Pakistan

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SUMMARY

Tissues that require a great supply of nutrients and possess high metabolic demands, such as lympho-hemopoietics tissues, are the first to be affected by protein malnutrition (PM). Thus, PM directly affects hemopoiesis and the production and function of immune cells. Consequently, malnourished individuals are more susceptible to infections. Mesenchymal stem cells (MSCs) have immunomodulatory properties and are important in the formation of lympho-hemopoietic stroma. Since an adequate supply of nutrients is essential to sustain stroma formation, which is mainly constituted of MSCs and differentiated cells originated from them, this study investigated whether PM would influence some biological and immunomodulatory aspects of MSCs. Two-month-old Balb/c mice were divided into control and malnourished groups receiving normoproteic or hypoproteic diets, respectively (12% and 2% of protein) for 28 days. MSCs obtained from control (MSCct) and malnourished (MSCmaln) animals were characterized. In addition, the proliferation rate and cell cycle protein expression were determined, but no differences in these parameters were observed. In order to evaluate whether PM affects the immunomodulatory properties of MSCs, the expression of NFκB and STAT-3, and the production of IL-1α, IL-1β, IL-6, IL-10, TGF-β and TNF-α by MSCs were assessed. MSCmaln expressed lower levels of NF-κB and the production of IL-1β, IL-6 and TGF-β was significantly influenced by PM. Furthermore, MSCct and MSCmaln culture supernatants affected lymphocyte and macrophage proliferation. However, MSCmaln did not reduce the production of IFN-γ nor stimulate the production of IL-10 in lymphocytes in the same manner as observed in MSCct. Overall, this study implied that PM modifies immunosuppressive properties of MSCs.

1. Introduction

Protein malnutrition (PM) is an abnormal physiological condition and the most common and widespread nutritional disorder that affects a large number of people around the world, especially young children in Asia, Latin America, the Near East and Africa [1,2]. PM is caused by an inadequate or poor utilization protein intake with reasonable caloric (energy) intake and can be associated or not with a low consumption of lipids, vitamins, and minerals [3,4]. PM disturbs homeostasis and it is clinically associated with metabolic alterations, histological and functional changes that may induce organ failure [5,6], and increased susceptibility to infections [4,7].

The first tissues to be affected by PM are those with high metabolic rates, such as lympho-hemopoietics [8,9]. PM decreases production of the blood cells, affecting the progression of cell cycle and the differentiation capability of hematopoietic stem cells. This can lead to hypoplasia as well as structural alterations in the bone marrow (BM), interfering with both innate and adaptive immunity, which results in increased susceptibility to infections [4].

The BM stromal compartment has long been recognized as a key structural component, acting in constructing and maintaining BM microenvironments, interacting with hematopoietic populations and influencing development of the immune response. The stromal compartment derives from common non-hemopoietic progenitor cells of mesenchymal origin, called mesenchymal stem cells (MSCs) [10,11].

* Corresponding author. Experimental Hematology Laboratory, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Avenida Lineu Prestes, 580 Bloco 17, Sao Paulo, SP, 05508-900, Brazil.
E-mail address: hemato@usp.br (R.A. Fock).

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MSCs are cells with high capability of self-renewal and classical differentiation into tissues of mesodermal origin such as osteocytes, adipocytes and chondrocytes [11]. MSCs have an important role in the BM microenvironment, regulating the hemopoietic niche and supporting hemopoiesis [12,13]. Moreover, MSCs have important immunomodulatory properties through the production and secretion of soluble factors, such as cytokines, chemokines, growth factors and by cell–cell contact [14]. MSCs have a marked plasticity capability: they exhibit both pro- and anti-inflammatory phenotypes and act in immune control regulation as well as in tissue repair and regeneration. Thus, MSCs can modulate many kinds of immune cells, including T cells, B cells, dendritic cells, and natural killer (NK) cells [15,16].

In recent years, an increasing number of studies have reported the effects of MSCs on hemopoiesis and their inhibitory effects on immune cells. However, there is scant information in the literature regarding the effects of malnourishment on MSCs. Available information indicates that MSCs can influence various physiological and pathophysiological processes, specially immune and inflammatory responses and that, in states of PM, innate and adaptive immunity are impaired. Therefore, this study aims to investigate whether PM also influences the immunomodulatory properties of BM MSCs.

2. Materials & methods

2.1. Animals and diets

Male mice of the BALB/c inbred strains of two-month-old were individually maintained at 22 ± 3 °C, and relative humidity at 55% ± 10%, with a regular 12-h light/dark cycle. Mice were fed with a control diet for 10 days in order to stabilize their body weight (adaptation period). After this period, mice were divided into two groups, which received either a normoproteic (control group) or a hypoproteic (malnourished group) diet for a period of 28 days (induction to malnourishment period). This study was approved by the Ethics Committee of the School of Pharmaceutical Sciences at the University of São Paulo.

Normoproteic (control) and hypoproteic diets were prepared in house. The mineral and vitamin mixtures were prepared following the American Institute of Nutrition for adult mice recommendations [17]. The protein source used was casein (>85% protein). Both diets contained 100 g kg^{-1} sucrose, 80 g kg^{-1} soybean oil, 10 g kg^{-1} fiber, 2.5 gk g^{-1} choline bitartrate, 1.5 g kg^{-1} L-methionine, 40 g kg^{-1} of mineral mixture and 10 g kg^{-1} of vitamin mixture. The control diet contained 120 g kg^{-1} casein and 636 g kg^{-1} cornstarch, while the malnourishment diet contained 20 g kg^{-1} casein and 736 g kg^{-1} cornstarch. With the exception of the protein and corn starch content, the two diets were identical and isocaloric, providing 1716.3 kJ/100 g. The final protein content of both diets was confirmed by the standard micro-Kjeldahl method [18].

Nutritional evaluation was performed by monitoring body weight, food consumption and protein intake every 48 h during the period of induction to malnourishment. The variation in body weight was calculated as a relative value between the body weight on the first day of induction to malnutrition and the last day of this period.

2.2. Blood

After 28 days of malnourishment induction, mice were euthanized and blood samples were collected for hematological evaluation and plasma was separated by centrifugation (1000 × g for 10 min at 4 °C). The concentrations of serum proteins, albumin, and pre-albumin were determined by the use of commercial kits (Labtest Diagnostica SA, Lagoa Santa, Brazil) and based on standard methods.

Blood samples were collected from both control and malnourished animals and preserved in EDTA (Sigma Aldrich, St. Louis, MO, USA) to avoid clot formation. Hemograms were obtained by loading blood samples into ABX Micros ABC Vet® equipment (Horiba ABX, Montpellier, France). The morphological and leukocyte differential analyses were performed on blood smears stained by May-Grünwald-Giemsa (Sigma Aldrich) technique.

2.3. Bone marrow cellularity, isolation and culture of bone marrow mesenchymal stem cells (MSCs)

BM cells were obtained by flushing femurs with Dulbecco's modified Eagle's medium containing low glucose (DMEM) (Cultilab, Campinas, Brazil) supplemented with 10% fetal calf serum (Cultilab, Campinas, Brazil), 1% Penicillin and Streptomycin (Sigma Aldrich) without glutamine. This resulting solution (MSC culture medium) was used to further culture MSCs. BM cellularity was determined by counting obtained cells using a Neubauer chamber and morphological and differential cell counts were performed on smears stained by May-Grünwald Giemsa. MSCs from control (MSCct) and malnourished (MSCmaln) mice were cultured and isolated based on the capacity of MSCs to adhere to plastic in a low glucose medium and characterized based on the methods previously described by Friedenstein et al. [10] and Caplan [11]. MSCs were cultured in MSC medium and maintained at 37 ± 0.2 °C, 5 ± 0.1% CO_2 and in a humidified atmosphere. Non-adherent cells were discarded by medium refreshment, which was replenished on days 3, 7, and 14 of cell culture. MSC growth was monitored every 2 days by bright field microscopy and MSC morphology was observed at days 3, 7, and 14 after seeding cells in culture flasks. After 14 days in culture, cells achieved 90% of confluence and were passaged. Therefore, MSCs at passage 2 were used for experimentation.

Cell confluency and CFU-F count were determined at days 3, 7, and 14 after seeding 5 × 10^5 MSCct or MSCmaln in 35 mm tissue culture plates (Corning, Tewksbury, USA). After 14 days in culture, CFU-F containing more than 50 cells were visualized and counted using an inverted microscope.

2.4. MSC characterization

The phenotypic MSC profile was determined by flow cytometry. MSCct and MSCmaln were trypsinized, centrifuged, and supernatants were discarded. Cell pellets were incubated with the following antibodies: anti-CD90.2 (FITC clone 30-H12), -Sca1 (FITC, clone D7), -CD13 (FITC, clone R3-242), -CD34 (FITC, clone RAM34), -CD45 (FITC, clone 30-F11), -CD14 (FITC, clone rmC5-3) and -CD271 (FITC, clone MLR2), all antibodies were purchased from Becton Dickinson Pharmingen, San Diego, CA, USA with exception of the CD271 which was purchased from Abcam, Cambridge, MA, USA. Once labeled, 1 × 10^4 cells were acquired by flow cytometry (FACScanto II®, BD, San Jose, USA). Data was analyzed and biomarker expression was quantified with FlowJo® 7.6 software (TreeStar, Ashland, USA).

The differentiation capacity of MSCs into osteoblasts, chondrocytes, and adipocytes was evaluated using commercial kits from R&D Systems (cat. no. SC010, R&D Systems, Abingdon, UK).

2.5. Assessment of MSC cell cycle and apoptosis

MSCct and MSCmaln were fixed in 4% paraformaldehyde (Sigma Aldrich), permeabilized with 0.1% of Triton X-100 (Sigma Aldrich), treated with RNase (BioRad, Philadelphia, USA) and labeled with Propidium Iodide Staining Solution (BD Pharmingen®). Once

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